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Membranous Material of Bovine Milk Fat Globules. II. Some Physical and Enzymic Properties of the Deoxycholate-Released Lipoproteins*

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ABSTRACT: Water-soluble particles of lipoprotein are released from the membrane of milk fat globules by sodium deoxycholate (DOC), leaving a layer of water-insoluble complexes composed of lipids and proteins, still attached to the globules. The lipoproteins released by DOC have a wide range in ultracentrifugal heterogeneity. Therefore, the population of lipoproteins was fractionated by ultracentrifugal flotation into three density classes: <1.13, 1.13–1.21, and >1.21 g/ml. Lipoproteins in each of these classes were analyzed ultracentrifugally. Sedimentation coefficients indicated that particle interaction occurred among the lipoproteins during the flotation procedure, and that both ionic strength of the flotation medium and the amount of lipid in the lipoproteins were factors in particle aggregation. DOC-released lipoproteins were precipitated irreversibly in an acidic medium; the maximum precipitation occurred at pH 3.8. Experiments to determine the solubility dependence of these lipoproteins on ionic strength were done at pH 7.0, using ammonium sulfate. Approximately 93% of the lipoproteins were salted out at 45% ammonium sulfate saturation; the process was reversible. Xanthine oxidase and alkaline phosphatase activities were assayed in fractions isolated, by ammonium sulfate precipitation, from the DOC-released lipoproteins. There was no marked precipitation of either enzyme, and it was concluded that these two proteins occur together in the lipoprotein particles. The distribution of the above enzymes between the water-soluble and the water-insoluble membranous fractions was determined. Both enzymes were localized principally in the water-soluble lipoproteins. This finding provides additional support for our previously proposed hypothesis that the membrane of the milk fat globule consists of two types of lipid–protein complexes distinguishable on the basis of solubility in water. A model in accordance with this hypothesis is compared to those previously proposed by other investigators.

A procedure for releasing water-soluble particles of lipoprotein (deoxycholate-released lipoproteins) from the membrane of intact milk fat globules by sodium deoxycholate (DOC)[1] was described in our preceding paper (Hayashi and Smith, 1965). These lipoproteins[2] account for approximately 45% of the protein and 67% of the phospholipids present in the original membrane. The fat globules remaining after the release of these lipoproteins were found to maintain their independent existence in the aqueous medium. To explain these observations, a tentative model of the structure of the membrane was proposed. In this, the water-soluble lipoproteins are regarded as being adsorbed on a structural matrix of water-insoluble complexes composed of lipids and proteins, which is in contact with the triglyceride core of the fat globule.

This paper reports studies aimed at finding methods to isolate a more homogeneous molecular species from the heterogeneous population of DOC-released lipo-

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[1] Abbreviation used: DOC, sodium deoxycholate.

[2] In this paper the term lipoproteins refers to the water-soluble particles composed of lipids and proteins, whereas the term lipid–protein complexes is used to denote either water-soluble or water-insoluble material composed of lipids and proteins.

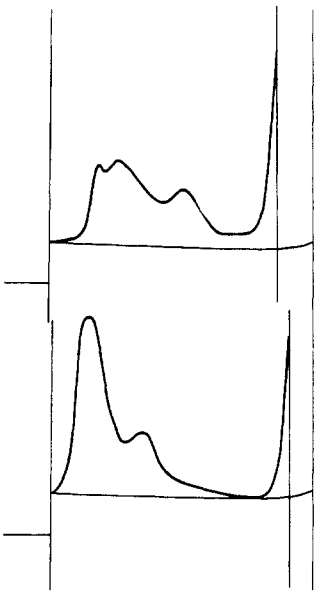

FIGURE 1: Sedimentation patterns of the deoxycholate-released lipoproteins in 0.0125 M Tris buffer at pH 8.5 at solvent densities 1.0031 g/ml (upper pattern) and 0.0540 g/ml (lower pattern). Densities were adjusted by diluting a concentrated lipoprotein solution with equal volumes of water or D_2O. The sedimentation runs were made in a Spinco analytical ultracentrifuge, Model E, at 42,040 rpm, 20 ± 1°, in double-sector cells. The patterns shown were obtained 96 min after the rotor attained operational speed. Sedimentation is toward the right.

proteins. Some physicochemical and enzymic properties of the DOC-released lipoproteins are described.

Experimental Procedures and Results

General Procedures

Preparation of Deoxycholate-Released Lipoproteins. The DOC-released lipoproteins were obtained according to procedure I described in the previous report (Hayashi and Smith, 1965). The source of the washed cream used was fresh uncooled milk pooled from several Holstein and Jersey cows.

Analytical Procedures. Sedimentation analyses were made with a Spinco Model E ultracentrifuge at 20 ± 1° in double-sector cells at 42,040 rpm. Solution densities were determined using 1-ml self-filling pycnometers at room temperature and a Mettler Gramomatic balance. The observed densities were corrected to d_4^{20}.

Protein contents were determined from Kjeldahl nitrogen according to the method of Ma and Zuazaga (1942). The conversion factor of 6.25 mg of protein/mg of nitrogen was used. In the solubility studies, relative protein concentrations were estimated spectrophotometrically from the absorbance at 280 mμ. Xanthine oxidase activity was assayed, using vanillin as substrate, essentially according to the method of Kuramoto *et al.* (1957). The arbitrary unit of enzymatic activity was the change in absorbance at 640 mμ/5 min under the specified conditions.

In the alkaline phosphatase assay, the method of Lowry *et al.* (1954) was modified by using 1 M ethanolamine instead of 2-amino-2-methyl-1-propanol as buffer at pH 9.9. The substrate was *p*-nitrophenyl phosphate. Absorbance at 410 mμ was followed spectrophotometrically at 30° for 10^3 sec. Pellets were homogenized with a Teflon tissue homogenizer prior to assay. The arbitrary unit of activity was an increase in absorbance of 1.00 at 410 mμ/second under the specified conditions.

Fractionation Procedures

As reported previously (Hayashi and Smith, 1965), the lipoproteins released from intact, washed cream globules by deoxycholate have a wide range in ultracentrifugal heterogeneity. A prerequisite for further characterization of these lipoproteins is their fractionation into more precisely defined subfractions. Attempts were made to fractionate these lipoproteins into density classes by ultracentrifugal flotation.

ηS vs. ρ Study. Information concerning the density of the predominant species in the population of the DOC-released lipoproteins was required in order to proceed with the ultracentrifugal subfractionation. This information was obtained from an ηS *vs.* ρ study made the following way. First, the DOC-released lipoproteins were centrifuged in a Spinco Rotor No. 30.2, at 79,000*g* for 12 hr at 4°, immediately after their elution from the gel-filtration column. This run concentrated the lipoproteins in the bottom milliliter of the solution in the preparative tubes. The lipoproteins were removed from the tubes with a capillary pipet. The isolated lipoproteins (not including the small pellet which formed during the run) are referred to as the concentrated DOC-released lipoproteins.

The concentrated DOC-released lipoproteins were analyzed ultracentrifugally at two solvent densities, namely, d_4^{20} of 1.0031 and 1.0540 g/ml. These analyses were made at equal lipoprotein concentration and equal ionic strength of the medium. Density adjustments were made by diluting the solution of concentrated DOC-released lipoprotein with an equal volume of water or heavy water (99.9% D_2O). The density range was limited to avoid excessive dilution.

Figure 1 presents the sedimentation velocity patterns obtained in 0.0125 M Tris chloride buffer at pH 8.5, 20 ± 1°, 96 min after the rotor had reached 42,040 rpm. Sedimentation coefficients for the fast and major peaks of the lipoproteins in the solvent density of 1.0031 g/ml were 8.20 and 4.21 S, respectively. The coefficients for the fast and major components of the lipoproteins in the solvent density of 1.0540 g/ml were 6.25 and 2.72 S, respectively.

The ηS *vs.* ρ plot in Figure 2 was constructed from the observed sedimentation coefficients given above. The values used for η, the coefficient of viscosity, were those for water and for a 0.5 mole fraction mixture of D_2O

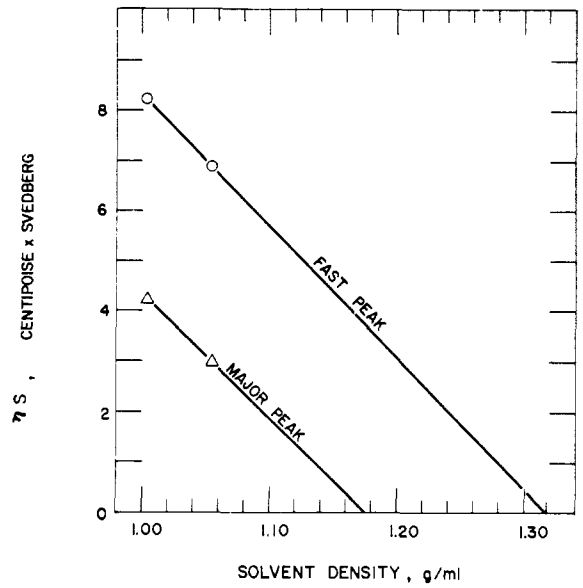

FIGURE 2: ηS *vs.* ρ plotted for the fast and major peaks observed for the deoxycholate-released lipoproteins at pH 8.5 in 0.0125 M Tris buffer. This plot was constructed from the data obtained from the sedimentation experiment depicted in Figure 1. The observed ηS are extrapolated to zero, and the solvent densities at this value are considered the average hydrated densities of the two prominent sedimenting groups. This plot shows that the deoxycholate-released lipoproteins are composed of particles with a wide range in density.

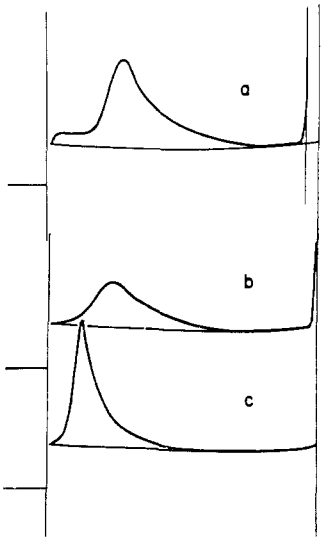

FIGURE 3: A comparison of the sedimentation patterns of the ultracentrifugal fractions isolated from the deoxycholate-released lipoproteins by the differential flotation method into the following density groups: less than 1.13 g/ml (pattern a), greater than 1.13 but less than 1.21 g/ml (pattern b), and greater than 1.21 g/ml (pattern c). These patterns were obtained in 0.02 M Tris buffer at pH 8.5, 20 ± 1°, 16 min after the rotor reached the operational speed of 42,040 rpm. Sedimentation is toward the right. It can be seen that after isolation, the sedimentation velocity observed for the low-density fraction, a, is greater than that for the high-density fraction, c.

in water [calculated from data in the Smithsonian Physical Tables (1954)]; these values at 20° were 1.005 and 1.10 centipoise, respectively. The solvent densities, ρ, were obtained by pycnometry and corrected to 20°.

Estimates of the density of the sedimenting components were made by extrapolating the experimental data to zero sedimentation velocity. The solvent density corresponding to zero ηS was considered the average hydrated density of the sedimenting group. The density for the group associated with the major peak was approximately 1.18 g/ml, and that for the fast peak approximately 1.32 g/ml. The ηS *vs.* ρ plot shows that the DOC-released lipoproteins consist of species covering a wide range in density, some of which approach the density of proteins.

Subfractionation of Deoxycholate-Released Lipoproteins. The population of DOC-released lipoproteins was fractionated into three density classes, namely, densities less than 1.13, densities between 1.13 and 1.21, and densities greater than 1.21 g/ml. This fractionation was accomplished by the ultracentrifugal flotation technique in two stages. For the first flotation run, the density of the solvent was adjusted to a d_4^{20} of 1.1274 g/ml by mixing the concentrated DOC-released lipoprotein solution with an equal volume of sodium bromide solution whose density, d_4^{20}, was 1.2537 g/ml. This mixture was centrifuged in a Spinco Rotor No. 30.2, at 79,000*g* for 12 hr at 4°. Under these conditions, the lipoproteins with densities less than that of the medium concentrated in the top milliliter portion of the solution in the preparative tubes and gelled. This elastic gel was transferred into a dialysis bag with a small spatula and a capillary pipet. When dialyzed at 4–5° against 0.02 M Tris at pH 8.5, the gelled lipoproteins went back into solution. Since the temperature during dialysis was the same as that at which gelation occurred, this transition was attributed to the lowering of ionic strength and not to melting. The dialyzed material was designated as the lipoproteins of density class less than 1.13 g/ml.

The lipoproteins having densities greater than that of the medium concentrated in the bottom milliliter portion of the solution and were collected with a capillary pipet. These lipoproteins then were subjected to a second flotation run in a solvent whose density was 1.2118 g/ml. The density adjustment was made by mixing one part of the pooled bottom portions from the first run with 0.4 part of a sodium bromide solution whose density, d_4^{20}, was 1.4154 g/ml. The resulting mixture was centrifuged in the Spinco Model L centrifuge, Rotor No. 30.2, at 79,000*g* for 24 hr at 4°. After this run, the successive milliliter layers of the solution in the prepara-

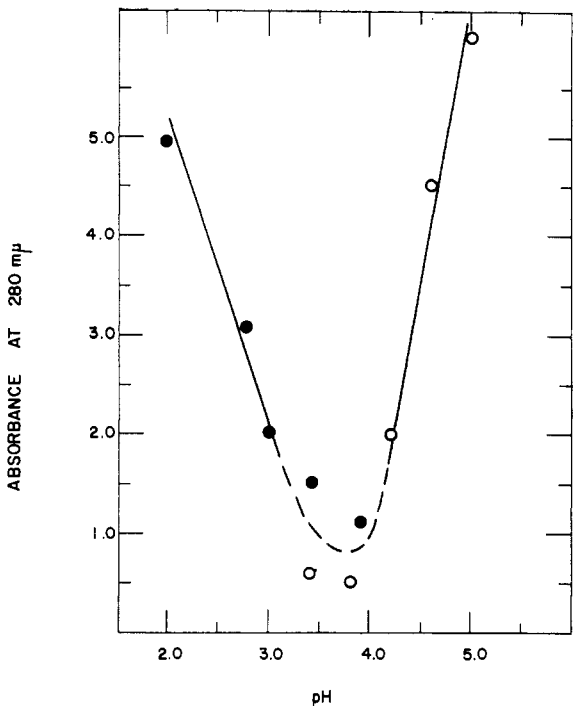

FIGURE 4: Effect of pH on the deoxycholate-released lipoproteins. Samples of a concentrated solution of the lipoproteins, at pH 8.5, were mixed with appropriate buffer solutions to reach the final pH in the acidic range, the ionic strength being held at 0.1 M with sodium chloride. The ordinate represents the absorbance at 280 mμ of the solution phases and the abscissa the final pH of the reaction mixtures after 32 hr at 0°. Solid dots and circles show data obtained with glycine hydrochloride-glycine buffers and acetic acid-sodium acetate buffers, respectively. Maximum precipitation occurred at pH 3.8. The precipitated lipoproteins could not be redissolved by readjusting the pH to 8.5.

tive tubes were removed individually with a capillary pipet. The top milliliter contained the lipoproteins with densities less than 1.21 but greater than 1.13 g/ml; the bottom milliliter contained lipoproteins with densities greater than 1.21 g/ml. These two fractions were dialyzed against 0.02 M Tris chloride buffer at pH 8.5 for approximately 24 hr at 4–5° to remove the sodium bromide. They then were designated, respectively, as density class 1.13–1.21 g/ml and density class greater than 1.21 g/ml.

Each of the three density classes of lipoproteins was analyzed ultracentrifugally. The ultracentrifugal analyses were made in 0.02 M Tris chloride buffer, pH 8.5, and the patterns were obtained 16 min after the rotor had reached the operational speed of 42,040 rpm.

The sedimentation pattern for the density class less than 1.13 g/ml (Figure 3a) shows a broad peak. The sedimentation coefficient of this peak was 24 S, considerably greater than the 8.2 S observed for the fastest component present in the unfractionated population (Figure 1). Evidently, the particle sizes of the species in this density class had increased under the conditions used for their isolation.

A broad distribution in sedimentation velocity is seen in the sedimentation pattern for the lipoproteins in the density class 1.13–1.21 g/ml, even though the species in this fraction had a comparatively narrow density range (Figure 3b). The sedimentation coefficient at the peak was 18 S. As can be noted from Figure 1, this density class contained the lipoprotein species which were most abundantly present in the unfractionated population. However, the major component in the unfractionated population migrated at the rate of 4.2 S.

The sedimentation coefficient for the peak in the class with density greater than 1.21 g/ml was 9.9 S (Figure 3c). In the unfractionated population (Figure 1), the fast component had a sedimentation coefficient of 8.2 S. Its average density of approximately 1.3 g/ml (Figure 2) indicated that the lipid content in these particles was relatively low. The small difference between the sedimentation coefficient for this density class and the coefficient for the fast component in the unfractionated population indicates there was little change in particle size as a result of the flotation procedure.

It became apparent that knowledge of the factors involved in the aggregation of the DOC-released lipoproteins was essential for further fractionation work. Therefore, the effects of pH and ionic strength on the solubility of the DOC-released lipoproteins were studied.

Effect of pH on DOC-Released Lipoproteins. To study the effect of pH on the solubility of lipoproteins at constant ionic strength, a series of reaction mixtures was prepared containing one volume of concentrated DOC-released lipoproteins (*ca.* 16 mg of protein/ml), one volume of an appropriate buffer to give the desired final pH, and $^1/_{10}$ volume of 2 M sodium chloride to provide a constant ionic environment. The buffer solutions used in preparing the above series all had an ionic strength of 0.05, and were as follows: glycine hydrochloride–glycine of pH 1.70, 2.30, 2.55, 3.00, and 3.50; acetic acid–sodium acetate of pH 3.45, 3.80, 4.15, 4.55, 4.90, and 5.50; potassium disodium hydrogen phosphate of pH 5.60, 6.25, 7.00, and 7.50; and Tris–hydrogen chloride of pH 8.1, 8.5, and 9.0. The ionic strength of the aqueous solvent was 0.1 M with respect to sodium chloride and 0.02 M with respect to the buffer.

The preparations were allowed to react for 32 hr at 0°, after which the pH of each was determined. Then any precipitates which had formed were separated from the solution phases by centrifugation at 0°. Since the precipitates were to be examined later for reversibility of the pH effect, the first centrifugation, by which most of each precipitate was recovered, was done at low speed to avoid compaction. Then the supernatant fluids, which were slightly turbid, were centrifuged at 8800*g* in a Spinco No. 30.2 rotor for 15 min, and the solution phases were then recovered. This operation was repeated until the solution phase of each sample had a constant absorbance at 280 mμ.

No precipitate formed in the samples whose pH was

greater than 5; the maximum precipitation occurred near pH 3.8. Typical results for that portion of the lipoprotein population remaining in solution are shown in Figure 4. Attempts to dissolve the precipitates by suspending them in various solvents such as water, the phosphate buffer at pH 7.0, and the Tris buffer at pH 8.5 were unsuccessful. Therefore, we concluded that the precipitation of the DOC-released lipoproteins occurring in an acidic environment is irreversible under the conditions of this experiment.[3]

From Figure 4 it is evident that the points near minimum absorbance did not coincide for the glycine hydrochloride–glycine and acetic acid–sodium acetate buffer systems. The absorbances in the glycine system were greater than in the acetate system. Furthermore, during the 32-hr reaction period, the pH shift of the glycine-buffered samples was approximately 0.4 pH unit toward neutrality, whereas the pH shift was negligible in the acetate-buffered series. The greater amount of lipoproteins remaining soluble in the glycine system than in the acetate system suggests possible interaction of glycine with the lipoproteins. This phenomenon has been observed with the β-lipoproteins of human serum (Oncley *et al.*, 1950).

Solubility Dependence on Ionic Strength at pH 7.0. In contrast to their irreversible precipitation in an acidic environment, DOC-released lipoproteins precipitated at neutral pH, by increasing the ionic strength of the solution, redissolved freely in an aqueous medium of low ionic strength. Accordingly, experiments to determine the nature of the solubility dependence of these lipoproteins on ionic strength were done, at pH 7.0, using ammonium sulfate.

Results of a typical experiment are presented in Figure 5. The logarithms of the solution absorbances at 280 mμ are plotted against the concentrations of the ammonium sulfate. The lipoprotein concentration was such (*ca.* 8 mg of protein/ml) that the salting-out phenomenon began when the ammonium sulfate concentration reached approximately 25% saturation. With further increases in the concentration of ammonium sulfate, the lipoproteins salted out progressively. The experimental points lie along two straight lines which intersect at about 45% ammonium sulfate saturation. The fact that there are two intersecting straight lines indicates the presence of at least two classes of soluble material. However, 93% of the DOC-released lipoproteins were salted out along the upper straight line.

The salting out under the above experimental conditions was found to be a reversible phenomenon. We therefore explored the possibility of fractionating the population of DOC-released lipoproteins by this means.

Enzymic Properties of Deoxycholate-Released Lipoproteins. Preliminary experiments showed that DOC-released lipoproteins possess both xanthine oxidase and alkaline phosphatase activities. Whether these enzymes are present uniquely or jointly in any given particle is of interest. To investigate this kind of homogeneity, the xanthine oxidase and alkaline phosphatase activities were assayed in various fractions precipitated from the DOC-released lipoproteins by increasing concentrations of ammonium sulfate.

In this work, samples of the DOC-released lipoproteins were mixed with ammonium sulfate solution, buffered at pH 7.0 with phosphate, to give the desired final ammonium sulfate concentrations. The mixtures were equilibrated for 24 hr at 0°, and the resulting precipitates were separated from the supernatant fluids by centrifugation. Both the precipitates and the supernatant fluids were dialyzed against 0.025 M Tris buffer, pH 8.5, to remove the ammonium sulfate. The dialyzed ma-

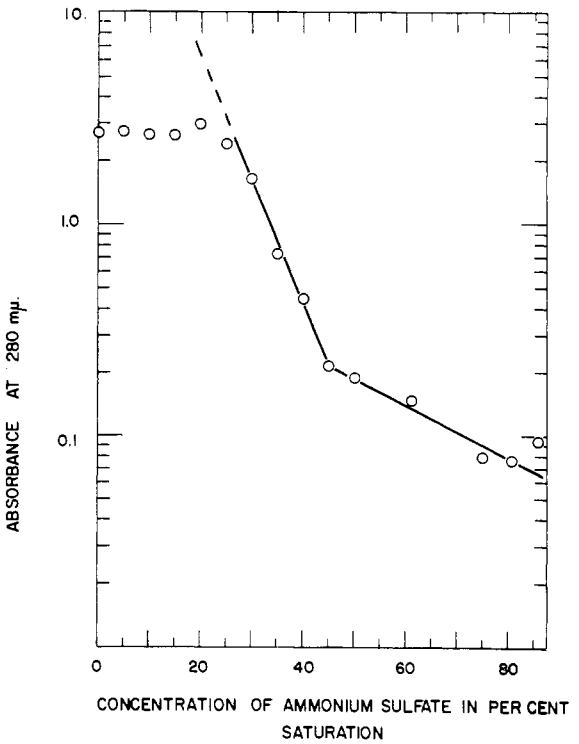

FIGURE 5: Salting-out behavior of the deoxycholate-released lipoproteins at pH 7.0. The ordinate represents the absorbance at 280 mμ of the solution phase in equilibrium with the precipitated lipoproteins at the various concentrations of ammonium sulfate in the reaction mixtures at pH 7.0; the abscissa represents the concentration of ammonium sulfate in per cent of saturation at 0°. Once precipitation begins, the experimental points for the major portion (*ca.* 93%) of the deoxycholate-released lipoproteins show a linear relationship between the logarithm of the solubility and the concentration of ammonium sulfate. This relationship is typical of many proteins. The precipitated lipoproteins redissolve readily upon the removal of ammonium sulfate by dialysis.

[3] One of the referees of this paper pointed out that Figure 4 was obtained for a system not in thermodynamic equilibrium and might be attributed to differences in the rate of protein denaturation. This may well be the process that occurred. The kinetics of denaturation may be dependent on species differences in the population in addition to pH.

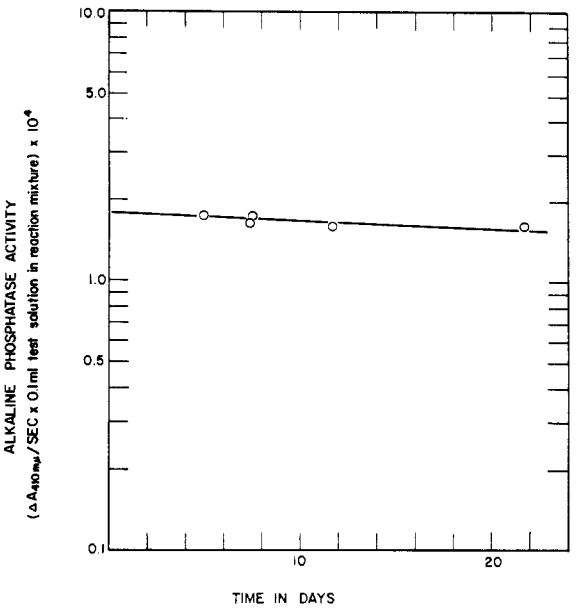

FIGURE 6: Decay of the alkaline phosphatase activity associated with the deoxycholate-released lipoproteins during storage time in 0.02 M Tris buffer, pH 8.5, at 2–5°. Samples were taken from the portion stored at the time intervals indicated. Assay method is described in General Procedures. For the period of observation (17 days) the activity fitted an exponential decay equation, and the loss in activity was at a rate of 0.7%/day.

TABLE I: Distribution of Activities of Alkaline Phosphatase and Xanthine Oxidase between Supernatant Fluids and Redissolved Precipitates Obtained from Deoxycholate-Released Lipoproteins with Various Concentrations of Ammonium Sulfate at pH 7.0.

% Saturation of $(NH_4)_2SO_4$	% of Original Activity	
	Alkaline Phosphatase	Xanthine Oxidase
	Supernatant Fluid	
0[a]	99	101
25	84	96
30	78	94
35	64	88
40	17	43
50[b]	5	5
	Redissolved Precipitate	
0[a]	1.8	0
25	1.9	0.3
30	12	1.7
35	40	23
40	73	45
50[b]	95	71

[a] Control sample was carried through all operations as were the other samples except for addition of ammonium sulfate. [b] Density of 50% saturated ammonium sulfate was such that the salted-out lipoproteins floated on centrifugation. For brevity, this flotation layer is classified with precipitated lipoproteins here and in the text.

terials were assayed for alkaline phosphatase and xanthine oxidase activities.

With increased ammonium sulfate concentration, the percentages of the original activities (at zero concentration of ammonium sulfate) of both alkaline phosphatase and xanthine oxidase decreased progressively in the supernatant fluid; this was accompanied by increases in the percentages of these activities in the redissolved precipitates (Table I). While 95% of the original alkaline phosphatase activity was recovered, only 71% of the original xanthine oxidase activity was found in the lipoproteins salted out by ammonium sulfate at 50% saturation. However, each of the fractions contained both alkaline phosphatase and xanthine oxidase.

Distribution of Alkaline Phosphatase and Xanthine Oxidase in the Membranous Fractions of the Milk Fat Globule. In the model of the membrane proposed earlier (Hayashi and Smith, 1965), the water-soluble lipoproteins were regarded as being adsorbed on a matrix of water-insoluble complexes composed of lipids and proteins. It was of interest to determine the distribution of enzymes between the water-soluble and water-insoluble components of the membrane. Accordingly, membranous material was prepared by treating washed cream with deoxycholate, as described previously. The washed cream and each of the fractions obtained were assayed for alkaline phosphatase and xanthine oxidase at intervals over a period of *ca.* 14 days. The observed activities were extrapolated to zero time. Figure 6 illustrates a typical decay curve for alkaline phosphatase. The activity of each enzyme, corrected to zero time, is summarized for each fraction in Table II.

The DOC-released lipoproteins contained about 40% of the total membranous protein and accounted for 63 and 81%, respectively, of the alkaline phosphatase and xanthine oxidase activities of the original washed cream. The portion of the membrane resisting the solubilizing action of the deoxycholate, designated as the residue (procedure I, step 7), contained 31% of the total membranous protein. However, it accounted for only 14 and 6%, respectively, of the alkaline phosphatase and xanthine oxidase activities of the original washed cream. Similarly these activities were low in the pellets relative to their protein contents. It is evident that these enzymes were localized principally in the DOC-released lipoproteins.

Discussion

The water-soluble lipoproteins released from the membrane of intact fat globules by sodium deoxy-

TABLE II: Distribution of Alkaline Phosphatase and Xanthine Oxidase Activities in the Membranous Fractions of Milk Fat Globules Prepared by Treating Washed Cream with Deoxycholate.

Fraction[a]	Total Protein[b] (mg)	% of Original Activity Alkaline Phosphatase	% of Original Activity Xanthine Oxidase
Washed cream	1109	100	100
Residue (step 7)	341	14	6.0
Pellet 1	84.4	0.5	2.8
Flotation layer 2	2.5	0.3	—
Pellet 2	127	0.6	7.4
Flotation layer 3	5.5	0.4	—
Pellet 3	120	1.2	6.9
Deoxycholate-released lipoproteins	428	63	81

[a] See text for description. [b] Protein contents were calculated from Kjeldahl nitrogen determinations, assuming that the protein contained 16% nitrogen. The values presented represent an average of three determinations.

cholate accounted for 40–45% of the original membranous proteins. Chemical analysis showed that the lipoproteins contained, on the average, approximately 50% lipid. That these particles, even though containing a high lipid content, were water soluble is suggestive of a similarity to lipoproteins of blood serum (Oncley *et al.*, 1950).

Because of the differences in density of the particles in the population of the DOC-released lipoproteins (Figure 1), the lipoproteins were subfractionated into three density classes. However, in this procedure, by necessity, the lipoproteins were subjected to high ionic strength. It is remarkable that the low-density lipoproteins, <1.13 g/ml, remained in solution when first exposed to the environment of high ionic strength, but gelled when concentrated by centrifugation in the same ionic medium. Furthermore, when the gelled lipoproteins were dialyzed to lower the ionic strength of the medium, the lipoproteins went back into solution. Thus, there were two factors implicated in the gelation: concentration of lipoproteins and ionic strength of the medium. The way these factors operated indicates that these low-density lipoproteins exist as a system capable of aggregation.

The intermediate density class of lipoproteins (1.13–1.21 g/ml) showed an increase in its sedimentation coefficient after isolation by the ultracentrifugal flotation technique; this also is indicative of aggregation of particles.

It is of interest to consider the phenomena of gelation and aggregation of the DOC-released lipoproteins in relation to the particle interactions necessary to maintain the structural integrity of the membrane. Conceivably, the lipoprotein particles could exist in equilibrium distribution between the surface of the native membrane and its aqueous environment. Assuming this, the experimental parameters governing the reversible precipitation of the DOC-released lipoproteins, *e.g.*, variation of ionic strength in a solvent at neutral pH, may also be the parameters under which the membrane may be disassembled in a way that would provide information on its structure. Moreover, the acidic environment which causes an irreversible precipitation of the DOC-released particles may also cause irreversible changes in the membrane of the intact fat globule.

Evidence that the solubility of the DOC-released lipoproteins is governed by the protein moiety is found in Figure 5. At pH 7, a straight-line relationship existed between the logarithm of the concentration of the lipoproteins and the concentration of ammonium sulfate. This relationship is characteristic of many homogeneous proteins (Cohn and Edsall, 1943).

Figure 5 provides a basis for explaining certain observations made by other workers. Successive washing of cream with distilled water was found by Zittle *et al.* (1956) to diminish successively activities of xanthine oxidase and alkaline phosphatase in the cream. However, Erickson *et al.* (1964) found that, when cream was washed with solution containing sodium chloride (0.15 M), the protein nitrogen in the washed cream was greater than when distilled water was used. In the present study, the DOC-released lipoproteins contained xanthine oxidase and alkaline phosphatase. Thus, the observations of Zittle *et al.* (1956) and Erickson *et al.* (1964) can be explained in terms of the relationship of the solubility of the DOC-released lipoproteins to the ionic strength of the wash water (Figure 5); that is, the increase in ionic strength inhibited the desorption of the membranous lipoproteins. The gelation of the low density lipoproteins (<1.13 g/ml) during the ultracentrifugal flotation also may be explained from the data in Figure 5. The linear extrapolation of the experimental points, indicated by the dotted line, approximates the solubility limit of the DOC-released lipoproteins at any particular ionic strength of the solvent. At the beginning of the ultracentrifugal flotation, the low density lipoproteins in dilute concentration were soluble in the ionic medium of 1.6 M sodium bromide. However, when these lipoproteins floated to the top of the solution in the preparative tubes they were concentrated beyond their solubility limit.

A major requirement to further the studies on the physicochemical properties of the DOC-released lipoproteins is to isolate well-defined classes of lipoprotein species from the original heterogeneous population. While the classification may be made by density groups, there remains the question of whether their previous contact with deoxycholate might induce migration of lipids from one lipoprotein species to another during the course of a given experiment. Other workers have reported that phospholipids migrate from the membrane of the fat globule to the milk plasma during agitation and heat treatment (Greenbank and Pallansch, 1961).

However, it is not clear whether phospholipids *per se*, or lipoproteins, migrated. The occurrence of any such migration may be detected if the ratio of the lipid mass to some specific property of the protein moiety, such as enzymic activity or amino acid composition, is followed for any given density class of lipoprotein. Therefore, the study of the distribution of xanthine oxidase and alkaline phosphatase activities in the DOC-released lipoproteins was made on the various fractions obtained by fractionally precipitating the DOC-released lipoproteins with ammonium sulfate. No exclusive precipitation of either xanthine oxidase or alkaline phosphatase was found in these fractions (see Table I). There was an apparent preferential precipitation of alkaline phosphatase; however, appreciable losses of xanthine oxidase activity in the redissolved precipitates were noted. Further work is necessary to ascertain whether or not preferential precipitation actually occurs. That xanthine oxidase is more labile than alkaline phosphatase when these enzymes are precipitated from washed cream buttermilk by ammonium sulfate and dialyzed has been noted by other investigators (Zittle *et al.*, 1956).

The evidence from the present study indicates that the protein moiety of the lipoproteins released by deoxycholate always contains both xanthine oxidase and alkaline phosphatase. In the results obtained by Morton (1954), the ratios of the activity of alkaline phosphatase to that of xanthine oxidase for the particles termed "milk microsomes" and the supernatant fluid isolated from buttermilk were essentially constant. Alexander and Lusena (1961) obtained six fractions by freezing washed cream and then treating the membranous material so obtained with sodium deoxycholate. All of their fractions contained both alkaline phosphatase and xanthine oxidase activities. However, some of their ultracentrifugally sedimented fractions showed marked enrichment of one or the other of these enzymes. Also, it is of interest that when Herald and Brunner (1957) removed the lipids from membranous material of milk fat globules with cold 35% ethanol in ether, they obtained protein fractions that were either soluble or insoluble in 0.02 M sodium chloride. Xanthine oxidase was found concentrated in the insoluble protein fraction and alkaline phosphatase in the soluble fraction. It is possible that the DOC-released lipoproteins of the present study are "packets" of metabolically related enzymes held together by lipids, and may be similar to ribosomes. Partial or complete delipidization of the packets could lead to the separation of these enzymes.

It is of interest to compare our hypothetical structure of the membrane with those presented by other investigators. King (1955) suggested that the various chemical constituents of the membrane are arranged in concentric layers around the triglyceride core. At the boundary of the core, phospholipids form a layer in which their less polar acyl groups are directed toward the triglycerides and their more polar groups toward the aqueous environment. The outer layers of the membrane were considered to be composed of proteins which interacted with the polar groups of the phospholipids. King's model does not provide a complete explanation for two interrelated facts found in the present studies: (1) the release of water-soluble lipoproteins which contain a substantial amount of phospholipids, triglycerides, and proteins, and (2) the retention by the triglyceride core of lipid–protein complexes which also contain phospholipids and proteins.

Morton (1954) suggested that the fat globule is surrounded by a continuous proteinaceous membrane on which is adsorbed a layer of globular particles which he termed "milk microsomes." However, these milk microsomes are particles which sedimented when centrifuged at 14,000*g* for 2 hr and represented the less abundant fraction of the membranous material; the more abundant fraction appeared in the supernatant fluid to which he gave little attention. Thus, while Morton's concept of an adsorbed layer of microsomes relates the membrane of the fat globules to the microsomes of the milk-secreting cells, it does not account quantitatively for all the membranous material.

In the model we propose, a layer composed of water-insoluble complexes containing lipids and proteins surrounds the triglyceride core of the fat globule. This layer may have a structure as depicted by King (1955) rather than the continuous protein membrane suggested by Morton (1954). The outer layer consists of adsorbed particles as in Morton's model, but for the most part these particles are water-soluble lipoproteins and are considered to be smaller than microsomes. That alkaline phosphatase and xanthine oxidase are predominantly localized in the water-soluble lipoproteins provides an additional criterion to distinguish the two layers in our model of the membrane.

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Optical Activity and Conformation of Carbohydrates. I. Optical Rotatory Dispersion Studies on Immunochemically Reactive Amino Sugars and Their Glycosides, Milk Oligosaccharides, Oligosaccharides of Glucose, and Blood Group Substances*

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ABSTRACT: Optical rotatory dispersion (ORD) spectra of a few selected carbohydrates and of the blood group A, B, and H substances are presented. The maltose and isomaltose series of oligosaccharides, the cyclic Schardinger dextrins, and amylose exhibit simple dispersion curves over the spectral region 589–200 mμ, with Drude dispersion wavelengths nearly the same for all but maltose. The blood group substances and Type XIV pneumococcal polysaccharide exhibit pronounced negative Cotton effects with troughs near 220 mμ. These Cotton effects are shown to be associated with the 2-acetamido (*N*-acetyl) group. The intensity of this Cotton effect is compared in a variety of 2-acetamido-2-deoxy sugars and in oligosaccharides containing *N*-acetylated sugars. The magnitude of the trough is about seventeen times greater in methyl β-(2-acetamido-2-deoxy)-D-glucoside than in ethyl β-(2-acetamido-2-deoxy)-D-galactoside. It is considerably diminished in the α anomers. Substitution of a β-D-galactosyl residue at carbon 3 or at carbon 4 in *N*-acetyl-D-glucosamine brings about an intensification of the trough magnitude. The deepest trough is observed with α-L-fucosyl-substituted *N*-acetyl-D-glucosamine as occurs in some of the milk oligosaccharides. An example of possible analytical utility is the requirement, from the ORD data, that the *N*-acetyl-D-glucosamine residues of Type XIV pneumococcal polysaccharide must be branched, in agreement with methylation studies of others.

Variations in the Cotton effect magnitude are interpreted using the octant rule and assuming a preferred orientation of the planar amide group with respect to the ring. The significance of these results is discussed, especially with respect to the relationship of preferred conformations of sugar rings to immunochemical specificity and antibody heterogeneity.

In the 148 years since Biot's discovery of the optical activity of cane sugar (Biot, 1817), a great number of measurements have been made of the optical rotation of carbohydrates. The majority of these were performed at a single wavelength (sodium D line or mercury green line). Attempts to understand the measured specific rotations in terms of the chemical structure have depended largely on the use of empirical rules such as those of van't Hoff (1875) and Hudson (1909). Lowry (1935) has reviewed the very considerable literature which had accumulated by that date. More recently, Kauzmann *et al.* (1961) have formulated rules for predicting the rotation of simple carbohydrates based on a summation of pairwise interactions.

This paper is concerned with the optical rotatory dispersion (ORD)[1] of a few selected carbohydrates and of the blood group A, B, and H substances to determine whether ORD reveals anything about ordered or periodic structures in polymeric carbohydrates and also

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[1] Abbreviations used in this work: ORD, optical rotatory dispersion; GNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; G, D-glucose, Gal, D-galactose.